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Human placental ribonuclease inhibitor abolishes both angiogenic and ribonucleolytic activities of angiogenin

(bovine pancreatic ribonuclease A/protein homology/regulation of angiogenesis)

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ABSTRACT **Human placental ribonuclease inhibitor (PRI) abolishes both the ribonucleolytic activity of angiogenin toward 28S and 18S rRNA and its angiogenic activity on the chicken embryo chorioallantoic membrane. Treatment of the angiogenin–PRI complex with *p*-hydroxymercuribenzoate releases enzymatically active angiogenin. Assays measuring competition between angiogenin and bovine pancreatic ribonuclease A for PRI reveal that binding of the inhibitor to angiogenin is extremely tight, with a K_i value well below 0.1 nM. The stability of the angiogenin–PRI complex was assessed by cation-exchange HPLC quantitation of free angiogenin. No significant dissociation was detected after 17 hr at 25°C in the presence of a large excess of bovine ribonuclease, which serves as a scavenger for free inhibitor. The results of these experiments, based on the predictive capacity of the angiogenin/RNase homology, suggest that PRI and related inhibitors may participate in the *in vivo* regulation of angiogenin and that this might have pharmacologic and/or therapeutic implications.**

The primary structure of angiogenin, a blood vessel-inducing protein from HT-29 human colon adenocarcinoma cells (1), is highly homologous to that of the pancreatic ribonucleases (RNases) (2, 3). Indeed, angiogenin exhibits ribonucleolytic activity, but its specificity differs distinctly from that of pancreatic RNase (4). Angiogenin catalyzes the limited cleavage of 28S and 18S rRNA; the resultant products are 100–500 nucleotides long. It does not exhibit significant activity in any of the standard pancreatic RNase assays that were examined (4).

From the point of view of protein chemistry, the mechanism of action of angiogenin—or, for that matter, of any other blood vessel-inducing protein—is not yet known. In this regard, however, its homology with RNase is most fortunate. It provides an excellent opportunity to benefit from the extensive detailed knowledge of RNase already on record, as well as a means to bypass conventional approaches to assess structure–function relationships. The predictive capacity of this knowledge encompasses (*i*) catalytically essential amino acid residues that might serve as mutagenic targets, (*ii*) specific reagents for chemical modification, and (*iii*) most pertinent to the present discussion, the potential of selective inhibitors.

Potent pancreatic RNase inhibitors have long been known to be present in mammalian tissues (see refs. 5 and 6 for reviews). One of these, isolated from human placenta, has been studied most extensively. It is an acid-labile, sulfhydryl-dependent protein with a molecular mass of 51,000 daltons. It is reported to bind to bovine pancreatic RNase A with a K_i of 0.3 nM (7). Human placental RNase inhibitor (PRI) has now been found to abolish both the biological and the enzymatic activities of angiogenin, thus further confirming that the angiogenin/RNase homology is functionally significant.

MATERIALS AND METHODS

Angiogenin was purified to homogeneity as described (1, 4) or by procedures to be published elsewhere. Human PRI was isolated by the method of Blackburn (8). Bovine pancreatic RNase A was a product of Cooper Biomedical (Malvern, PA). Calf liver rRNA (28S and 18S) was purchased from Pharmacia, and *p*-hydroxymercuribenzoate (*p*HMB), uridylyl(3',5')adenosine (UpA), hen egg white lysozyme, and human serum albumin (HSA) were obtained from Sigma.

Angiogenin and RNase A concentrations were determined as described (4). PRI concentrations were measured by titration with RNase A (8). RNase A activity toward UpA was measured spectrophotometrically (9). Assay mixtures contained 0.2 mM dinucleotide, 1 mM EDTA, and 10 μg of HSA per ml in 0.1 M Mes/0.1 M NaCl, pH 6. The decrease in absorbance at 280 nm was continuously monitored at 25°C with a Gilford model 250 spectrophotometer.

Activity of angiogenin toward rRNA was measured as described (4). Angiogenesis activity was assessed by a modification (1) of the chicken embryo chorioallantoic membrane assay of Knighton *et al.* (10). PRI used in chorioallantoic membrane assays was desalted in a Centricon-10 microconcentrator (Amicon). Inhibitor [stored in either 20 mM Tris/150 mM NaCl, pH 7.5, or 100 mM sodium acetate/3 M NaCl, pH 5.0, in both cases containing 1 mM EDTA, 5 mM dithiothreitol, and 15% (vol/vol) glycerol] was added to sterile deionized water that had been degassed and then saturated with nitrogen. This solution was concentrated by centrifugation in the microconcentrator according to the manufacturer's instructions. Inhibitor prepared by this procedure was fully active in the assay of Blackburn (8).

Carboxymethyl cation-exchange HPLC was performed with a Synchropak CM300 column (250 × 4.1 mm; SynChrom, Linden, IN), utilizing a Waters Associates liquid chromatography system in conjunction with a Hewlett–Packard 3390A integrator. Elution was achieved with a 10-min linear gradient from 220 to 620 mM NaCl in 20 mM sodium phosphate (pH 7.0) at a flow rate of 1.0 ml/min at ambient temperature. Column effluents were monitored at 214 nm.

RESULTS

Inhibition of Ribonucleolytic Activity of Angiogenin by PRI. Angiogenin catalyzes the cleavage of both 28S and 18S rRNA, forming characteristic products that are 100–500 nucleotides in length (Fig. 1, lane 2). Addition of 1.2 molar equivalents of PRI to angiogenin completely inhibits this

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Abbreviations: PRI, human placental ribonuclease inhibitor; *p*HMB, *p*-hydroxymercuribenzoate; UpA, uridylyl(3',5')adenosine; HSA, human serum albumin.

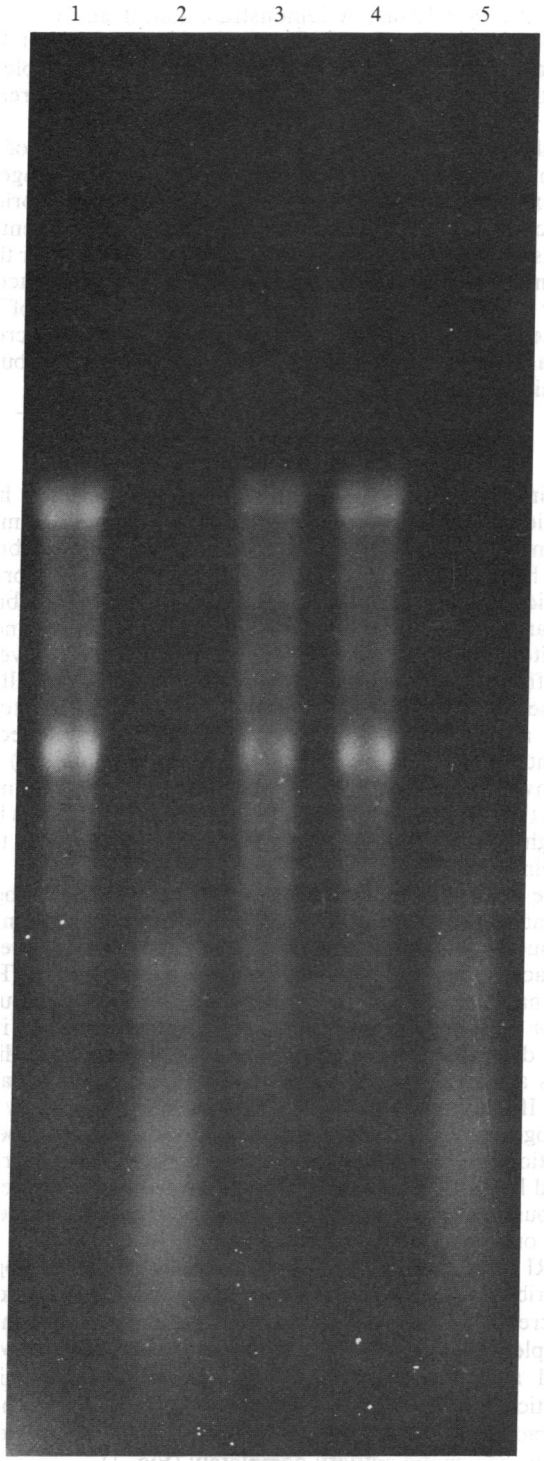

FIG. 1. Effect of human PRI on ribonucleolytic activity of angiogenin. RNA (12 μg) was incubated with or without angiogenin and/or inhibitor at 37°C in 33 mM Hepes/33 mM NaCl, pH 7.5. After 30 min, the reaction was terminated as described (4), and samples were electrophoresed in a 1.1% agarose gel. The gel was stained with ethidium bromide and photographed under ultraviolet illumination. Lane 1: control RNA sample. Lanes 2–4: samples containing 0.8 μM angiogenin, a mixture of 0.96 μM PRI and 0.8 μM angiogenin, and 0.96 μM PRI, respectively. Lane 5: sample containing 0.8 μM angiogenin recovered from angiogenin–PRI complex by treatment with 1 mM *p*HMB for 30 min at room temperature, followed by chromatography on Mono S and C_{18} HPLC columns as described (4).

activity (Fig. 1, lane 3). Since 1 mM *p*HMB rapidly dissociates the RNase A–PRI complex, releasing active RNase A and inactive PRI (7), the dissociability of the angiogenin–PRI complex was similarly tested with *p*HMB. The formation of free angiogenin was monitored by cation-exchange HPLC. A peak was eluted at the same time as native angiogenin, indicating that *p*HMB indeed releases angiogenin from the complex. Untreated complex remains intact during the course of the HPLC procedure and is eluted well before angiogenin (see below). The amount of angiogenin recovered represents 70% of that added initially and has full enzymatic activity (Fig. 1, lane 5).

Competition Between Angiogenin and RNase A for PRI. The competition between angiogenin and RNase A for the placental inhibitor was examined by means of an enzymatic assay. The dinucleotide UpA is not cleaved by angiogenin at a detectable rate (4), but it is an excellent substrate for RNase A. Therefore it was used to assess the partitioning of PRI between the two proteins. The activity measured reveals directly the amount of RNase A not bound to PRI. Assay mixtures contained various amounts of angiogenin, 0.27 nM RNase A, and 0.20 nM PRI. The RNase A and angiogenin were added to the assay buffer first, and the mixture was preincubated with inhibitor for 5 min at 25°C. The enzymatic reaction was then initiated by addition of substrate.

The amount of PRI employed is sufficient to inhibit 70% of the potential RNase A activity. When angiogenin is present it binds to PRI, which is therefore unavailable to inhibit RNase A. Hence, increasing concentrations of angiogenin progressively decrease RNase inhibition; at 5.8 nM angiogenin there is essentially no inhibition at all (Fig. 2). Lysozyme (40 nM) closely similar to both angiogenin and RNase in size and basicity, has no effect.

These competition assays examine the relative degrees of binding of PRI to angiogenin and RNase A upon addition of the inhibitor to a mixture of the two enzymes (see *Discussion*). An estimate of the K_i value for binding of PRI to angiogenin was obtained by a modified procedure in which PRI and angiogenin were preincubated for 10 min, followed by addition of RNase A and measurement of UpA cleavage. Since dissociation of the PRI–angiogenin complex is extremely slow (see below), this assay determines the amount of free inhibitor remaining after incubation of PRI with angiogenin alone. At an angiogenin/PRI ratio of 1.2, there is no detectable PRI inhibition of RNase activity—i.e., no free PRI. Thus, all of the PRI must be complexed to angiogenin

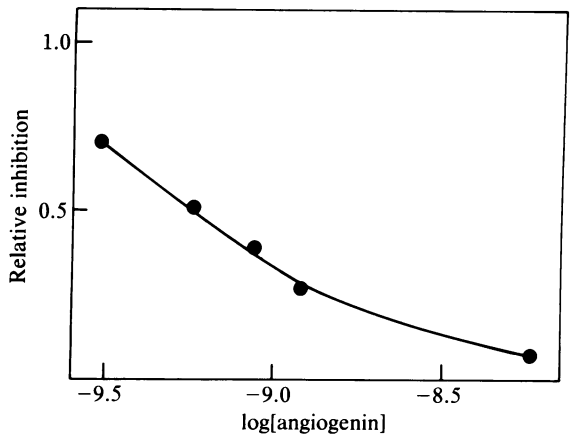

FIG. 2. Effect of angiogenin concentration on PRI inhibition of RNase A activity toward UpA. Relative inhibition is defined as $(V_0 - V_A)/(V_0 - V_R)$, where V_0 denotes velocity in the absence of PRI, and velocities in the presence of PRI are denoted V_R (no angiogenin added) or V_A (angiogenin added). Assay mixtures contained 0.2 mM UpA, 0.27 nM RNase A, 0.2 nM PRI, and 10 μg of HSA per ml in 0.1 M Mes/0.1 M NaCl/1 mM EDTA, pH 6.0. For further details, see text.

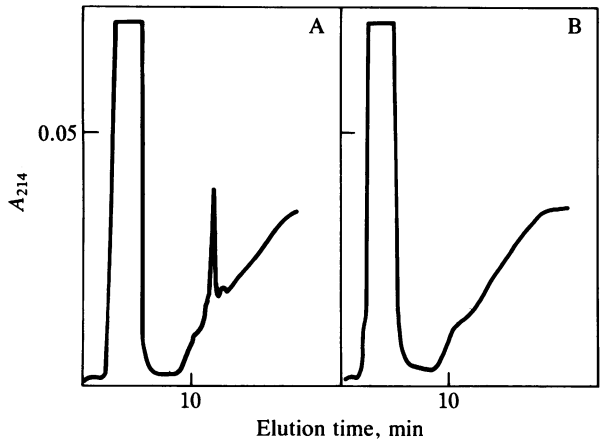

FIG. 3. Carboxymethyl cation-exchange HPLC of 0.64 μg of angiogenin (*A*) and a mixture of 0.64 μg of angiogenin and 12 μg of PRI (*B*). Samples were in 1 ml of 0.1 M Tris (pH 7) containing 1 mM EDTA and 10 μg of HSA and were incubated at room temperature for 10 min prior to injection. Elution was achieved as described in *Materials and Methods*.

under these conditions, indicating extremely tight binding, with a $K_i < 0.1$ nM.

Stability of the Angiogenin–PRI Complex. The stability of the angiogenin–PRI complex was assessed by cation-exchange HPLC (Fig. 3). In the system employed, neither the inhibitor nor buffer alone results in any peaks in the region of the chromatogram where free angiogenin is eluted (≈11 min). When a mixture containing angiogenin and excess PRI is injected onto the column, there is no peak at the position of free angiogenin. Given the acidic pI of PRI (7), under the conditions of this chromatography the angiogenin–PRI complex would not be expected to bind to the column. Indeed, treatment of unretained material with 1 mM *p*HMB generates free angiogenin, as indicated by the presence of a characteristic peak on rechromatography.

The complete absence of a free angiogenin peak in Fig. 3*B* shows that significant dissociation does not occur during the 3–4 min required for the complex to pass through the column. The stability of the complex over a longer period of time was determined by HPLC measurement of free angiogenin concentrations at various times after addition of a 32-fold excess of RNase A. In this case the RNase A serves to scavenge any free inhibitor released from the angiogenin–PRI complex. The amount of free angiogenin present would then be a direct measure of the extent of dissociation. Fig. 2 suggests that RNase A should act as an effective scavenger under the conditions employed. Measurements at 0.7, 6.5, and 17 hr after addition of RNase A demonstrate that, in all cases, less than 5% of the angiogenin is present in the form of free protein. Thus, dissociation of the angiogenin–PRI complex is an extremely slow process with a $t_{1/2}$ significantly greater than 1 day.

Table 1. PRI inhibits angiogenesis activity of human angiogenin

Sample	% positives (total number of eggs)		
	Exp. 1	Exp. 2	Exp. 3
Angiogenin	58 (24)	52 (54)	62 (13)
PRI	17 (29)	33 (48)	17 (12)
Angiogenin + PRI	15 (26)	25 (52)	7 (14)

Assays were performed on chicken embryo chorioallantoic membrane as described (1). Data for experiments 1 and 2 represent composites of results obtained in two and three sets of assays, respectively. Between 10 and 20 eggs were utilized for each of the three experimental groups within each set. The following amounts of angiogenin and PRI were employed: Exp. 1, 75 ng and 2 μg, respectively; Exp. 2, 46 ng and 700 ng; Exp. 3, 25 ng and 180 ng. Angiogenin was dialyzed vs. water prior to use. PRI was desalted using a Centricon microconcentrator. This procedure resulted in >1000-fold dilution of the buffer in which PRI stock solutions were stored.

Inhibition of Angiogenic Activity by PRI. The effect of the placental inhibitor on the angiogenic activity of angiogenin was examined by means of the chicken embryo chorioallantoic membrane assay, which evaluates the extent of neovascularization induced by test samples 3 days after their implantation (Table 1). Angiogenin consistently produces a highly significant, positive response. When an excess of PRI is mixed with the angiogenin prior to assay, activity decreases to a level indistinguishable from that observed with buffer or inhibitor controls.

DISCUSSION

Protein inhibitors of pancreatic and related RNases have been identified in the cytoplasm of a wide variety of mammalian tissues (5, 6). Purified preparations of such inhibitors from human placenta (7), bovine brain (11), and porcine thyroid and liver (12) inhibit bovine RNase A with subnanomolar K_i values, making them by far the most potent known inhibitors of this enzyme. These proteins have also proved to be effective inhibitors of non-pancreatic intracellular RNases; as a result, it has recently become customary to use them as reagents to increase yields of higher molecular weight proteins in *in vitro* translation systems (13) and improve the quality of RNA isolated from tissue homogenates (14). Hence, the *in vivo* role of these inhibitors has been thought to be related to control of mRNA turnover and, thus, protein synthesis (15).

The primary sequence of angiogenin is highly homologous to that of pancreatic RNase (2, 3). In fact, angiogenin is a ribonucleolytic enzyme (4). We have therefore examined its interaction with the human placental RNase inhibitor (PRI). Originally isolated from medium conditioned by human tumor cells, angiogenin is not a tumor-specific protein: it has been detected in a variety of other sources, where it likely plays a role in normal and/or pathological neovascularization. If inhibitors such as PRI were to interact strongly with angiogenin, this might represent a hitherto unsuspected function for them: regulation of angiogenesis. Moreover, this could lead to avenues of exploration for the management of various pathological conditions characterized by or dependent on abnormal neovascularization.

PRI is indeed a potent antagonist of both the angiogenic and ribonucleolytic activities of angiogenin. As with bovine pancreatic RNase A (6), this inhibition is reversible, and the complex can be readily dissociated by addition of *p*HMB to yield angiogenin that is active and chromatographically identical to the native protein. The stoichiometry of the interaction is 1:1; a slight molar excess of PRI is sufficient to inhibit enzymatic activity completely (Fig. 1).

Specific substrates of angiogenin have not been identified as yet, and since our present assay system for the measurement of its ribonucleolytic activity (4) employs relatively high enzyme and substrate concentrations, its utility for the determination of nanomolar or lower inhibition constants is greatly restricted. For that reason the K_i value for the PRI–angiogenin interaction was estimated indirectly by competition experiments based on the capacity of angiogenin to prevent PRI inhibition of RNase A activity. This gave a value much less than 0.1 nM, since addition of 0.24 nM angiogenin to 0.2 nM PRI effectively eliminated any inhibition of RNase added subsequently. In the absence of angiogenin, inhibition of 0.27 nM RNase is virtually stoichiometric, which suggests, moreover, that the K_i of PRI for RNase is at least an order of

magnitude less than the value of 0.3 nM reported previously (7).

The competition experiments also provide information concerning the relative affinities of PRI for angiogenin vs. RNase A. When PRI is added to a mixture of angiogenin and RNase A, equal concentrations of the angiogenin–PRI and RNase A–PRI complexes are formed at a total angiogenin concentration twice that of total RNase A (Fig. 2). Under these conditions the concentrations of free angiogenin and RNase A are calculated to be 0.48 and 0.17 nM, respectively. If this assay mixture were at equilibrium, these results would indicate that the K_i value of PRI for angiogenin is 2.8-fold higher than that for RNase A. However, data obtained by HPLC for angiogenin (cited above) and for RNase A show that this is not an equilibrium situation, since the dissociation rates for both complexes with PRI are extremely slow. Thus, the partitioning of inhibitor between angiogenin and RNase more likely reflects the relative rates of association rather than the relative K_i values.

Blackburn and coworkers (6, 16–18) have studied the structural basis for the interaction between PRI and RNase A by means of proteolytic and chemical modifications and found that carboxymethylation of Lys-41 of RNase A substantially reduces binding strength, indicating an important role for this residue. They further suggested three contact regions for PRI in the three-dimensional structure of RNase A: the areas containing (*i*) Lys-7, Lys-41, Pro-42, Val-43, Lys-91, Tyr-92, and Pro-93; (*ii*) Lys-31 and -37; and (*iii*) Lys-61 and adjacent residues. In these regions angiogenin and RNase A are remarkably similar, with identical residues at positions corresponding to Lys-41, Pro-93, Lys-61, and Asn-62 and similar residues at positions corresponding to Lys-7 (His), Val-43 (Ile), Tyr-92 (Trp), Lys-31 (Arg), and Gln-60 (Asn). Hence, both proteins might be expected to bind PRI with comparable avidity. Indeed, the strength of the angiogenin–PRI interaction provides experimental support that the overall three-dimensional structures of angiogenin and pancreatic RNase are closely similar, as already suggested by energy-minimization procedures (19).

The establishment of the chemical structure of angiogenin and the detection of its extensive homology with pancreatic RNase were critical in recognizing that PRI is a powerful inhibitor of angiogenin. This inhibition affects both the biological and enzymatic activities of angiogenin and may have important mechanistic, physiologic, and pharmacologic implications. It is consistent with the hypothesis that these two actions of angiogenin are interrelated, as suggested previously by the simultaneous loss of both activities upon carboxymethylation by bromoacetate at pH 5.5 (4). Further, it raises the possibility that PRI may play a role in the *in vivo* regulation of angiogenin. As noted above, the angiogenin–PRI interaction likely involves regions of angiogenin that are separated widely in the three-dimensional structure, many of them outside the active center. Thus, conservation of residues necessary for enzymatic activity alone probably cannot account for the strength of the interaction. This implies that the capacity of angiogenin to bind PRI has been maintained independently during evolution. It would therefore seem plausible that binding by PRI and related inhibitors reflects a physiologically relevant control mechanism that might have pharmacologic and therapeutic potential.

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